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RESEARCH ARTICLE

Rapid and Sensitive Diagnosis of Cattle Anaplasmosis by Loop-Mediated Isothermal Amplification (LAMP)

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A B S T R A C T

Anaplasmosis is a disease by an intraerythrocytic rickettsial pathogen in cattle, which mainly caused by *Anaplasma marginale* (*A. marginale*). Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification technology by which the target DNA can be efficiently amplified with high specificity and sensitivity under isothermal conditions. In this study, a LAMP method was developed for rapid and easy diagnosis of cattle Anaplasmosis. Three groups of specific primers targeting major surface protein 5 gene (*msp5*), were designed and optimized, followed by confirmation of the specificity and sensitivity of LAMP assay. The result demonstrated that *A. marginale*-positive sample was efficiently amplified by LAMP assay and no presence of LAMP amplicon in the four blood-borne parasites control samples, *Besnoitia besnoiti*, *Trypanosome sp*, *Toxoplasma gondii* and *Besnoitia bigemina*, was confirmed by either visual inspection or agarose gel electrophoresis. One femtogram of total genomic DNA from *A. marginale*-positive blood was qualified to be detected by LAMP assay, which was 100 times more sensitive than that of conventional PCR. The data demonstrated that LAMP assay was a potential of high specificity, sensitivity and time-effectiveness to diagnose Anaplasmosis in cattle herds, especially for undeveloped countries.

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INTRODUCTION

Anaplasmosis, an arthropod-borne, economically important disease in dairy and beef cattle, is endemic worldwide in most tropical, subtropical and many temperate countries (Aggarwal and Bannon, 2014). Anaplasmosis is mainly caused by *Anaplasma marginale* (*A. marginale*), resulting in decreased production due to lower milk yields, lower weight, abortion, even death (Aubry and Geale, 2011). *A. marginale* was first described by Theiler in South Africa in 1909 which the scientific name is based on its staining characteristics and location within host erythrocytes, i.e. "marginal points" in bovine erythrocytes. Theiler isolated *A. central* in 1910, which is often located centrally with host erythrocytes. Anaplasma can cause a fatal infectious disease of cattle in South Africa, which later was recognized as the first richettsial pathogen (Palmer, 2009). *A. marginale* is an infectious but non-contagious disease, and transmitted to cattle biologically by ticks or mechanically by flies or blood-contaminated fomites like contaminated needles, ear tagging devices, unclean instruments (Aguirre *et al.*, 1994), sometimes be spread by transplacental transmission (Aubry and Geale, 2011). *A. marginale*, being considered as an important pathogen for *Anaplasmosis*, results in fever, abortion, recurring parasitemic peaks, cyclic anemia and occasional death, especially in animals over two years of old. The infections and subsequent transmission in crossbred and exotic breeds of cattle by *A. marginale* in the tropics and subtropics often result in high morbidity and mortality (Singh *et al.*, 2012), being heavy economic burden in cattle industry.

Proper treatment of a disease begins based on the rapid and correct detection of pathogens or diagnosis of the disease. 16S rRNA (Lew *et al.*, 2003), heat-shock protein (GroEL) (Lew *et al.*, 2003), MSP1a (Lew *et al.*, 2002), MSP4 (Ma *et al.*, 2011) and MSP5 (Corona *et al.*, 2009) are often chosen to be the targets for detection of *A. marginale*. Of note, *msp5* gene, 633bp in length in the form of a single copy in the genome of *A. Marginale*, encoding a 19kDa protein, which was defined by

immunoblots with monoclonal antibody ANAF16C1, is the most conserved among all recognized species of anaplasma, including *A. marginale*, *A. centrale*, and *A. Ovis* (Visser *et al.*, 1992; Strik *et al.*, 2007; Ybanez *et al.*, 2013). The traditional detection methods targeting MSP5 of *A. marginale* include competitive enzyme-linked immunosorbent assay (cELISA) (Strik *et al.*, 2007), competitive inhibition ELISA (ciELISA) assay (Ni *et al.*, 2011), nested PCR (Torioni de Echaide *et al.*, 1998), real-time PCR (Picoloto *et al.*, 2010) and semi-nested PCR assay (Singh *et al.*, 2012). All of these methods each represent several limitations, such as varied sensitivity or labor-cost or time-cost. Moreover, the sophisticated equipments and skilled personnel may be required for the selected assays above. In 2000, Notomi and his colleagues developed a method to amplify DNA under isothermal conditions, usually between 60 and 65°C, termed Loop-mediated isothermal amplification (LAMP), which was of high specificity, efficiency and rapidity (Notomi *et al.*, 2000). It does not require sophisticated instruments, which is especially applicable in developing countries. Now it has been intensively applied to detect parasites, bacteria or viruses infecting human or animals (see the details in the reviews) (Parida *et al.*, 2008; Dhama *et al.*, 2014) (For further information, the reader is referred to read other excellent reports). Hundreds of application of LAMP in clinical detection and diagnosis have been reported since the latest reviews (Dhama *et al.*, 2014; Li and Macdonald, 2015) were published. However, the application of LAMP to diagnosis of cattle Anaplasmosis, especially as for *A. marginale*, remains to known whether it could be sensitive and effective as its performance for other pathogens in human or animals. Thus, we designed primers specific for the conserved *msp5* gene of cattle *A. marginale* in an attempt to evaluate the potential of the LAMP as a simple, rapid and sensitive diagnosis assay for cattle Anaplasmosis as to provide the guidance for the treatment or prevention of disease.

MATERIALS AND METHODS

Samples: The genomic DNA of whole blood from cattle infected by *A. marginale* was extracted using Genomic DNA extraction kit according to the manufacturer's instruction (TIANamp Genomic DNA Kit, IANGEN, Beijing, China) and stored at -20℃ until use. The samples of *Besnoitia besnoiti* (*B. besnoitia*), *Trypanosome sp*, *Toxoplasma gondii* (*T. gondii*) and *Besnoitia bigemina* (*B. bigemina*) were kindly provided by Dr. Wang in Preventive Veterinary Medicine, Parasite laboratory of College of Animal Science & Veterinary Medicine, Heilongjiang Bayi Agricultural University.

LAMP and conventional PCR primers specific for cattle Anaplasmosis: LAMP primers specific for Cattle *A. marginale* were designed based on the published sequence of *msp5* gene (GenBank accession number M93392.1). Total 3 groups (a, b, c) of primers were designed using PrimerExploer online server (http://primerexplorer.jp/e/). Three pairs of the outer primers were used for conventional PCR assay. Sequence of primers and related information is shown as Table 1.

Conventional PCR assay: PCR were conducted in a 25μL mixture containing 1μM outer primers, 1μL sample DNA, PCR 2×Master Mix (Thermo Scientific Fermentas, USA) and reaction was set as described elsewhere except annealing at 58°C for 1min, followed by 0.8% agarose gel electrophoresis (Corona *et al.*, 2009).

LAMP assay: LAMP reaction was performed as per manufacturer's instructions (New England Biolabs, Inc., MA). Briefly, the reaction was performed in a 25μL reaction mixture containing 0.4μM outer primers, 1.6μM inner primers , 0.8μM loop primers, 1μL of genomic DNA, 1mM of $MgSO_4$, 1mM dNTP, 1mM Betain, 2.5μL 10×Thermopol buffer, and 1U of Bst DNA polymerase (New England Biolabs, Inc., MA). Twenty microliter of mineral oil (Sigma-Aldrich, St. Louis, MO) was added to cover the surface of reaction mixture. Finally, 0.5μL SYBR GREEN I dye (Sigma-Aldrich, St. Louis, MO) was added into the center of inner cap of PCR tube. The mixture solution was incubated at 63°C for 20min, followed by centrifuge at 1000×g for 1min and shake.. The LAMP products were visualized under brighter field and UV-illuminator at 365nm, respectively. Alternatively, LAMP products were analyzed by agarose gel electrophoresis.

Optimization of LAMP assay reaction time: The optimal reaction time was determined based on the color change of reaction products at the time point of 10, 20, 30, 40, 50 or 60min, followed LAMP products were analyzed by electrophoresis on a 0.8% agarose gel, respectively.

Evaluation of LAMP: The specificity of LAMP for cattle Anaplasmosis was evaluated by comparison of amplification by using DNA from *B. besnoiti*, *Trypanosome sp*, *T. gondii* and *B. bigemina*. The sensitivity of LAMP for cattle Anaplasmosis was evaluated by comparing with conventional PCR by using 10-serial dilutions of DNA (10^{-1} to 10^{-7}) as templates, which was extracted from the positive blood sample infected by cattle Anaplasmosis using Total Genomic DNA extract kit as mentioned above. The LAMP assay was performed upon the optimized setting above.

RESULTS

Selection of outer primers: To determine the best primers for LAMP assay, three groups of outer primers (a, b, c) respectively was used to perform conventional PCR assay. Group A and group b primers qualified the amplification of DNA fragment of around 276bp, however, group c primers failed to complete the amplification. Furthermore, the DNA band amplified by group b primers was significantly brighter in visual inspection than that of group a (Fig. 1A). LAMP with the primers from each of three groups (a, b, c) was carried on. A distinct ladder-like DNA bands by either group a or group b primers was observed, whereas, group c primers failed to complete the amplification (Fig. 1B). Therefore, group b primers were assigned to develop a LAMP assay for the diagnosis of cattle Anaplasmosis.

Table 1: Primers designed for LAMP and conventional PCR in this study for the detection of cattle Anaplasmosis

Name	Primer(5′-3′)	Position	Length
MSP5-F3a †	ACCTTCTGCTGTTCGTTG	143	18
MSP5-B3a	GAGAAGCCATGCCTAACTC	400	19
MSP5-FIPa(F1c+F2)‡	GTTGAAAGACGCGGAGGCTGCGAGAGGTTTACCACTTC		38
MSP5-BIPa(B1c+B2)	CCGTCAGTAGCGGCGATTTGTACTTACAGGCTGAGAAGC		39
MSP5-LFa ¶	TGCCCTCACTTACAACTTCG	256	20
MSP5-LBa	CGGCAAGCACATGTTGGTA	324	19
MSP5-F3b	ACCTTCTGCTGTTCGTTG	143	18
MSP5-B3b	GAGAAGCCATGCCTAACTC	400	19
MSP5-FIPb(F1c+F2)	GTTGAAAGACGCGGAGGCTAAATCGGCGAGAGGTTTAC		38
MSP5-BIPb(B1c+B2)	CCGTCAGTAGCGGCGATTTGTACTTACAGGCTGAGAAGC		39
MSP5-LFb	TGCCCTCACTTACAACTTCG	256	20
MSP5-LBb	CGGCAAGCACATGTTGGTA	324	19
MSP5-F3c	CGTTGGGGTGTGATAGATG	102	19
MSP5-B3c	GTACTTACAGGCTGAGAAGC	369	20
MSP5-FIPc(F1c+F2)	GCCCTCACTTACAACTTCGGAAGGGGTACTCCTATGTGAACA		42
MSP5-BIPc(B1c+B2)	ATAGCCTCCGCGTCTTTCAACAAAATCGCCGCTACTGAC		39
MSP5-LFc	GGTAAACCTCTCGCCGATT	231	19
MSP5-LBc	GGTTAATCACGAGGGGGTC	282	19

Each group included 3 pairs of primers: outer, inner, and loop primers. †The forward outer primers were named as MSP5-F3a, MSP5-F3b and MSP5-F3c, the backward outer primers were named as MSP5-B3a, MSP5-B3b and MSP5-B3c. ‡The forward inner primers were named as MSP5-FIPa, MSP5-FIPb and MSP5-FIPc, the backward inner primers were named as MSP5-BIPa, MSP5-BIPb and MSP5-BIPc. ¶The loop primers were designed to accelerate the reaction, which contained the forward loop primers (MAP5-LFa, MSP5-LFb, MSP5-LFc) and the backward loop primers (MSP5-LBa, MSP5-LBb, MSP5-LBc).

Optimization of reaction time for LAMP assay: To optimize the performance of the LAMP assay, the incubation of reaction mixture was set for 10, 20, 30, 40, 50 or 60min. After incubation, the addition of SBYR GREEN I dye facilitated visual inspection. A color change from orange to green under brighter field among all six positive samples was observed except negative control, even though incubation reached to 60min (Fig. 2A). In addition, clear green fluorescence in all six reaction samples under UV365nm presented at varied incubation period. However, no clear green fluorescence showed in negative control (Fig. 2B). The all six positive LAMP amplicons presented ladder-like DNA pattern on agarose gel electrophoresis (Fig. 3).

Evaluation of LAMP assay for cattle Anaplasmosis: LAMP reaction under optimal condition and conventional PCR was performed with genomic DNA from *B. besonitia*, *Trypanosome sp*, *T. gondii* and *B. bigemina* to evaluate the specificity of LAMP assay. The DNA from four blood-borne parasites *B. besonitia*, *Trypanosome sp*, *T. gondii* and *B. bigemina* as templates in both PCR and LAMP assay failed to get the amplicon, in contrast, the amplification was confirmed in either LAMP (Fig. 4B) or conventional PCR (Fig. 4A) with the genomic DNA of cattle *A. marginale*. The sensitivity of LAMP assay for the cattle Anaplasmosis was determined by comparing with conventional PCR using 10-fold serial dilutions (10^0 to 10^{-8}) of 0.1μg total genomic DNA from cattle *A. marginale* as templates. The results showed that the ability of conventional PCR to complete the amplification under the lowest concentration of template was 10^{-6} of original genomic DNA, however, LAMP assay reached to 10^{-8} dilution, which was 100times more sensitive than that of conventional PCR assay (Fig. 5).

DISCUSSION

LAMP assay developed in this study demonstrated its high sensitivity, specificity, and time-effectiveness compared to conventional methods. The result suggested that the LAMP assay was high potential of detection and diagnosis of Anaplasmosis, since *msp5* gene is conserved among all recognized species of Anaplasma, including *A. marginale*, *A. centrale*, and *A. Ovis* (Visser *et al*., 1992). Besides, LAMP assay may be be helpful in the diagnosis and subsequent control of disease when Anaplasma is endemic in local area. Moreover, the determination of LAMP diagnosis can be determined by color change under UV-illumination or brighter field. Thus LAMP is most effective, applicable, and practical tools to detect or diagnose the pathogens in humans or animals, especially in the developing countries.

LAMP is more rapid, economical and suitable for clinical diagnosis of cattle Anaplasmosis than conventional diagnostic method (Li and Macdonald, 2015). It does not require sophisticated equipments, expensive reagents or skilled personnel for LAMP assay. LAMP reaction can be carried on in water bath, which is cost-effectiveness and easy operation, especially applicable in clinic. Comparatively, conventional cultivation of pathogens, serodiagnosis (Knowles *et al.*, 1996; Ramos *et al.*, 2014) and PCR (Torioni de Echaide *et al.*, 1998; Schotthoefer *et al.*, 2013), nested-PCR or Real-time qPCR (Picoloto *et al.*, 2010) requires specific and expensive instruments and qualified operator. Furthermore, methods above themself in comparison to the LAMP are time-consuming and complicated, which requires skilled operator to conduct it (Wang *et al.*, 2014).

MSP5 is a conserved protein of *A. marginale*, which can be detected in either acute or chronic infection by multiple species of anaplasma with McAb ANAF16C1 (Visser *et al.*, 1992). Analyses based on *msp1* and *msp5* of *A. marginale* isolated in Philippine revealed that *msp5* was a monophyletic lineage and was phylogenetically associated with the strains isolated in Brazilian or China (Ybanez *et al.*, 2013). It is of potential to be a molecular target for the detection or diagnosis for Anaplasmosis due to its high conservativeness (Strik *et al.*, 2007). Of note, Ma and his colleagues (2012) developed a LAMP assay based on *msp4* gene for detection of *A. ovis* (Ma *et al.*, 2011). In this study, the conserved region in *msp5* gene was chosen to be the target to develop a rapid and sensitive LAMP method to diagnose Anaplasma in cattle.

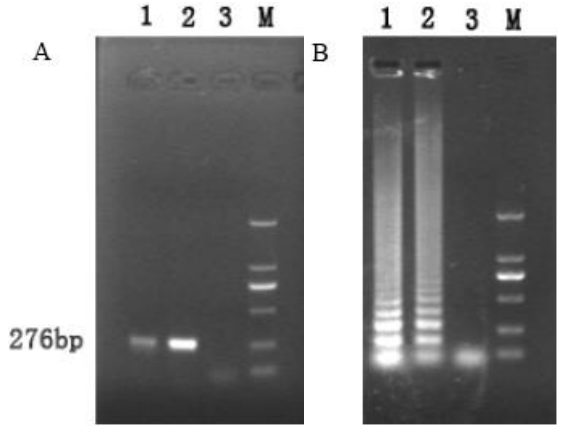

Fig. 1: Selection of primers with genomic DNA of bovine anaplasmosis as template. A) Conventional PCR assay by the outer primers and B) LAMP assay by three groups of primers. Lane 1: primers of group a; Lane 2: primers of group b; Lane 3: primers of group c; Lane M: DNA marker (DL2000).

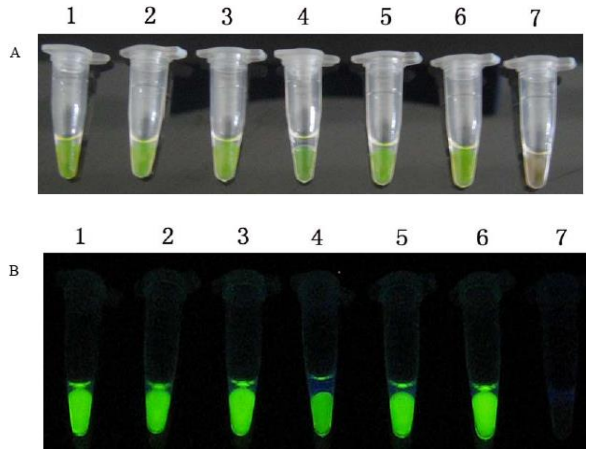

Fig. 2: Optimization of LAMP reaction time determined by visualization of LAMP products under brighter field (Panel A) and UV-illumination (Panel B). Lane 1-6: LAMP products with the varied incubation period: 10, 20, 30, 40, 50, 60 min respectively; Lane 7: negative control.

Though LAMP assay has been intensively applied to detect hundreds of pathogens in human beings or animals, the majority of applications often focused on the detection of bacteria (Zhuang *et al.*, 2014; Xia *et al.*, 2014) and viruses (Yang *et al.*, 2014), otherwise parasitic or rickettsial pathogens (For further information, the reader is referred to read other excellent report). Recently, RT-LAMP or LAMP assay was developed to detect tick-borne encephalitis virus and to detect cattle trypanosomiasis in southern Zambia, indicating that LAMP assay was a sensitive, specific, rapid and easy-to-operate method for the clinical diagnosis (Hayasaka *et al.*, 2013). In addition, Detection of Malaria by LAMP method was evaluated by comparison with microscopy, nested PCR, and quantitative PCR (qPCR), suggesting that sensitivity of LAMP assay for Malaria in a remote Ugandan clinic was much higher than that by microscopy inspection, nested PCR and qPCR (Hopkins *et al.*, 2013)., but similar to that by single-well nested PCR in a United Kingdom reference laboratory (Polley *et al.*, 2013). Furthermore, Pan *et al*. (2011) reported that a LAMP assay based on *MSP2* gene was developed to detect *anaplasma phagocytophilum*, suggesting a rapid, cost-saving method for *A. phagocytophilum* (Pan *et al.*, 2011).

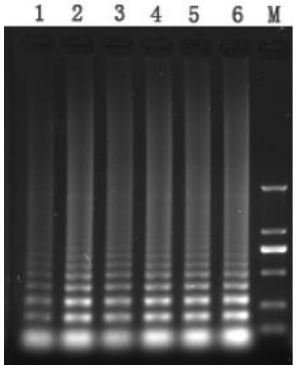

Fig. 3: Optimization of LAMP reaction time. Lane 1-6: LAMP products of different reaction time 10, 20, 30, 40, 50 and 60 min respectively; Lane M: DNA Marker (DL2000).

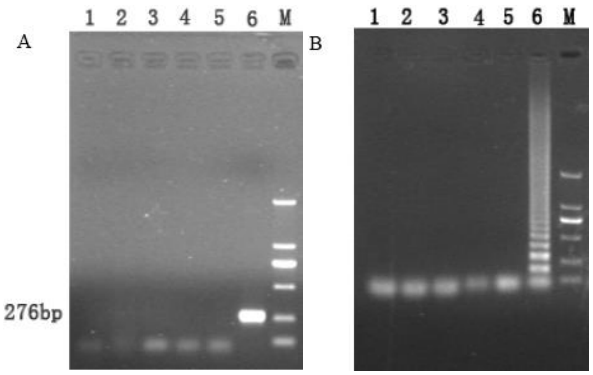

Fig. 4: Specificity of LAMP assay. Panel A: PCR assay with four blood-borne parasites and bovine Anaplasmosis genomic DNA. Panel B: LAMP assay with four blood-borne parasites and bovine Anaplasmosis genomic DNA. Lane 1: *B.besonitia* DNA; Lane 2: *Trypanosomiasis* DNA; Lane 3: *Toxoplasma* DNA; Lane 4: *B.bigemina* DNA; Lane 5: Negative Control; Lane 6: Bovine *Anaplasmosis* DNA; Lane M: DNA Marker (DL2000).

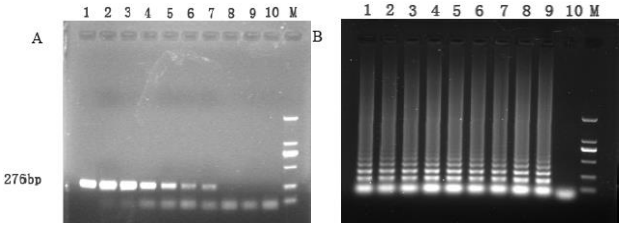

Fig. 5: Comparative sensitivity of conventional PCR (Panel A) and LAMP assay (Panel B). Lane 1-9: PCR or LAMP products with bovine Anaplasmosis DNA sample as template at 10^{0}, 10^{-1}, 10^{-2} to10^{-8} dilutions, respectively; Lane 10: negative control; Lane M: DNA Marker (DL2000).

In LAMP reaction mixture in this study, mineral oil was added to avoid volatilization and to segregate mixture solution with SYBR GREEN I dye prior to the incubation, in which may reduce aerosol and false positive. The LAMP method developed in this study demonstrated the same specificity as conventional PCR to differentiate four blood-borne parasites. However, LAMP in detecting *A. marginale* was 100 times more sensitive than that of conventional PCR. Our LAMP assay is expected to be further evaluated by testing the clinical samples in comparison with the conventional PCR aiming to provide the guidance for the treatment, regulation or even eradication program.

Conclusions: In this study, a loop-mediated isothermal amplification for conserved *msp5* gene of *A. marginale*

was developed for rapid diagnosis of cattle Anaplasmosis. The specificity and sensitivity of LAMP assay were evaluated by comparison to conventional PCR, which demonstrated that LAMP was specific and 100 times sensitive as conventional PCR for detection of genomic DNA of *A. marginale*. Moreover, the amplification was confirmed by visual inspection under UV-illumination or brighter field, instead of by agarose electrophoresis, by which simplified the diagnosis of cattle Anaplasmosis in clinic.

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Author's contribution: HTJ, YLZ, XYL and JL executed the experiment and XBW prepared the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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